



Proteomic identification of adhesive on a bone sculpture-inlaid wooden artifact from the Xiaohe Cemetery, Xinjiang, China



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ABSTRACT

With the emergence and progress of composite tools in the Middle Stone Age, the adhesive became one of the most widely used materials by early human societies. However, the precise composition identification of adhesive in archaeological remains is a real analytical challenge, because the adhesive mainly consists of organic materials that are susceptible to decay during burial process. Of particular interest is to know which animal/plant species were being exploited for glue manufacturing other than for food. The arid climate of the Xiaohe Cemetery, located in Taklamakan Desert, northwestern China, provides favorable conditions for the preservation of organic residues. A bone sculpture-inlaid wooden artifact was collected from the Xiaohe Cemetery, with some semi-transparent yellowish adhesive exposed due to the detachment of an inlaid bone sculpture. In this paper, micro samples of the adhesive were scraped for FTIR (Fourier Transform Infrared Spectroscopy, primary examination) and subsequent proteomic analysis to determine the proteinous component(s) and precise origin(s). The identified tryptic peptides match most closely to known bovine collagen markers, suggesting that this adhesive was an animal glue made from cattle. These results reveal the diverse utilizations of cattle in the Xiaohe Cemetery, which provided not only meat, milk, hides, sinews and dung, but also leftover parts for manufacturing adhesive. This is the earliest evidence of adhesive identified in China up to our knowledge, which sheds light on adhesive development around 3500 years ago.

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1. Introduction

According to the definition of the Encyclopedia Britannica, "any substance that is capable of holding materials together in a functional manner by surface attachment that resists separation" can be called adhesive (Pike, 2014). The history of adhesives is closely related to that of humankind. With the emergence and development of composite tools in the Middle Stone Age, the adhesive became one of the most widely used materials by early human societies. Hafting residue has been recorded throughout the world in a wide range of substances, for example, at the European sites of Campitello Quarry (ca. 200 ka) and Konigsau (ca. 80 ka) in the form of birch bark tar (Koller et al., 2001; Mazza et al., 2006), at the

South African sites of Diepkloof (ca. 56 ± 10 ka) and Sibudu (ca. 70 ka) in the form of conifer resin and Acacia gum respectively (Charrié-Duhaut et al., 2013; Wadley et al., 2009), at the Western Asian site of Umm-el-Tlel (ca. 70 ka) in the form of bitumen (Boëda et al., 2008), etc. In addition to early use for hafting, adhesive was also widely applied in painting, decoration, lacquer, bows and arrows, building as well as pottery repairing (Calvano et al., 2011; Chiavari and Mazzeo, 1999; Luo et al., 2012; Mitkidou et al., 2008; Regert, 2004; Wei et al., 2011; Yang et al., 2010). However, the precise composition identification of adhesive in artistic and archaeological remains is a real analytical challenge, because the adhesive mainly consists of organic materials that are susceptible to decay during burial. Of particular interest is to know which animal/plant species were being exploited for glue manufacturing other than for food.

Various adhesive materials were used in ancient times, including animal glue, egg, casein, blood, plant resin and gum,

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starch, pitch, tar, wax, oil, etc., which require different analytical protocols. Micro-chemical analysis and staining methods can be useful to detect the presence of different categories of organic substances and suitable for primary examination (Dallongeville et al., 2013; Fan et al., 1996). Spectroscopy methods, such as Infrared Spectroscopy and Raman Spectroscopy, which are non- or micro-destructive, can obtain general information of the composition and need further analysis (Edwards et al., 2009). Starch grain analysis is a common archaeobotanical method newly developed for the study of starch-rich remains. This method sometimes can identify the precise plant origin of starch (Yang et al., 2012), but sometimes incapable due to similar morphological characteristics of starch grains among the same plant genus (Zhang et al., 2011).

The most commonly used methods for adhesive analysis are chromatographic approaches, including gas-phase chromatography and liquid-phase chromatography (coupled with mass spectrometer). GC/MS, Py-GC/MS and HPLC have been applied to characterize oil, natural resin, gum, tar and wax (Modugno et al., 2006; Regert, 2004; Scott et al., 2009; Wei et al., 2011) and also to distinguish different kinds of proteinaceous media, such as animal glue, egg white, egg yolk and casein, through amino acid and peptide analysis (Fremout et al., 2009; Su et al., 2005). However, the precise species identification of proteinaceous adhesive remains difficult for these methods. Immunoassay techniques, like ELISA, are specific and informative, but they are limited to targeted protein (Scott et al., 2009). More recently, proteomic approaches, based on high resolution mass spectrometry, have been utilized and rapidly developed for identification of archaeological protein binders due to their high sensitivity and accuracy which can determine the precise origin of the binder using very low sample amount (Chambery et al., 2009; Dallongeville et al., 2011b, 2013; Fremout et al., 2010; Fremout et al., 2011; Leo et al., 2009).

In China, the studies on adhesive materials are rare and mostly focus on the binders of painted artifacts and polychromy, such as lacquer objects (Wei et al., 2011), polychromy terracotta army and jar (An, 2012; Chiavari and Mazzeo, 1999; Wei et al., 2012), mural paintings (Li, 1995; Su et al., 2005), colored paintings of architectural buildings (Yang et al., 2010), etc. Furthermore, Cheng et al. (2008) and Luo et al. (2012) have studied the binding agents on Chinese turquoise-inlaid bronze swords dated to the East Zhou period (770–256 BC). However, adhesives applied in other kinds of relics have not attracted sufficient attention yet. On the other hand, these studies are restricted to the historical period, and no research on prehistoric adhesive materials is available so far to the best of our knowledge.

It is to be noted that the arid climate in the Lop Nur area (Xinjiang, China) provides advantaged conditions for the preservation of organic residue. Dated to 1980–1450 BC (CRAIXAR, 2007; Li, 2010; Li et al., 2010), the Xiaohe Cemetery is the earliest Bronze Age site ever known in this area. A bone sculpture-inlaid wooden artifact, described as a staff, was collected from the Xiaohe Cemetery, with some transparent yellowish adhesive exposed because the inlaid bone sculpture dropped off. In this study, FTIR (Fourier Transform Infrared Spectroscopy) was initially used to evaluate the presence of protein in this adhesive. Subsequently a proteomic approach was employed to analyze the adhesive collected from a prehistoric context for the first time in China, in order to identify its precise origin, to reveal the related information about the utilization of natural resources and the production of adhesive in the Xiaohe Culture.

2. Sample background

2.1. Site description

The Xiaohe Cemetery ($40^{\circ}20'11''N$, $88^{\circ}40'20.3''E$) is a 7.75 m high mound of sand with five layers of burials, about 60 km south of the Peacock River in the Taklamakan Desert of northwestern China (Fig. 1). As a representative site of the Xiaohe Culture, the Xiaohe Cemetery was named by Chinese State Administration of Cultural Heritage as one of China's top ten archaeological discoveries in 2004. This site was first investigated in 1934 by a Swedish archaeologist Folke Bergman, and then comprehensively and professionally excavated by Xinjiang Cultural Relics and Archaeology Institute between 2002 and 2005. A total of 167 graves were discovered with well-preserved mummies, their dressed textiles and accompanied artifacts thanks to the extremely arid climate and saline soil. The discovered phallus-posts and vulvae-posts, wooden sculptures, rods, staffs, ox skulls, sheep bones, clothes, leather, bronze, bread wheat, common millet and other artifacts, imply that agriculture, husbandry, handicraft and religious beliefs played important roles in the Xiaohe people's life, and also provide important and rich materials for the research of prehistoric Eastern and Western communication (Amat, 2011; CRAIXAR, 2004, 2007).

2.2. Archaeological sample

Many bone sculpture-inlaid wooden artifacts, described as staffs, have been found in the Xiaohe Cemetery. Staffs are considered as important weapons of shamanism (Amat, 2011). They were

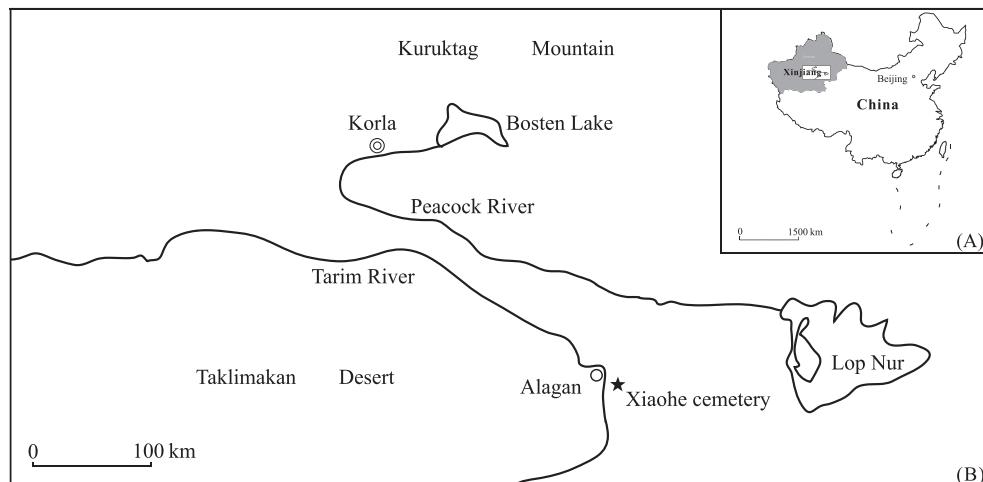


Fig. 1. Location of the Xiaohe Cemetery in Xinjiang. (A) Location of Xinjiang in China. (B) Location of the Xiaohe Cemetery.

frequently found standing at the tomb occupiers' head and feet respectively, indicated by tomb M24 (Fig. 2), whose occupier was regarded as a shaman (Liu, 2009). The production process of staffs is quite complicated: firstly, a wood pole is cut and divided into three sections; after that, two long vertical grooves are chiseled on the flat obverse of the upper section with two bone sculptures inlaid, and feathers are affixed on the reverse; finally, ephedra twigs are tied onto the middle section, which is then wrapped with mane and twine ropes (CRAIXAR, 2007). Because it was not permitted to sample intact staffs, one without mane or ropes was selected for analysis (Fig. 3A), whose unilateral inlaid bone sculpture was detached with some adhesive exposed (Fig. 3B). In order to avoid contamination, the interior adhesive was sampled after removal of the surface materials. The adhesive appeared to be homogeneous and semi-transparent yellowish (Fig. 3C), which was collected for FTIR and subsequent proteomic analysis to determine the biological origin(s).

3. Analytical protocols

3.1. FTIR analysis

FTIR analysis was employed to characterize major constituents of the adhesive and guide further analysis. The sample was analyzed as KBr micropellets with a Nicolet 6700 (Thermo Scientific) FTIR spectrometer working in a transmission mode. Spectra were acquired over the range of 4000–400 cm⁻¹ with a resolution

of 4 cm⁻¹ and 32 scans per spectrum. The software OMNIC 8.0 was applied to deal with the data.

3.2. LC/MS/MS analysis

- (1) Protein extraction. 100 µl of extracting solution (Tris–HCl, pH 8.0, 10 mM dithiothreitol, 10% sodium dodecylsulfate and 0.0025% bromophenol blue) was added to approximately 10 mg of archaeological sample. The mixture was subjected to ultrasonic baths (3 × 15 min) followed by incubation for 1 h at 56 °C. Then sonicated again for 15 min and centrifuged for 15 min at 12,000 g.
- (2) SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). 45 µl of the supernatant was mixed with 5 µl of glycerol, heated at 95 °C for 5 min, cooled to room temperature, and loaded onto the gel (5% stacking gel, 6% separating gel) with 25 µl of mixture each well. The electrophoresis apparatus was initially connected to an 80 V power supply and then switched to a 120 V power supply when the sample arrived at the separating gel. As the sample ran on the separating gel for about 3 cm, the apparatus was turned off and gel removed. A microwave-assisted Coomassie Blue staining protocol was followed. The gel immersed in the staining solution (0.25% Coomassie Blue w/v, 50% ethanol, 10% acetic acid) was incubated in the microwave oven at medium-low heat for 45 s followed by slowly shaking for 10 min. Then the staining solution was dumped. The gel was

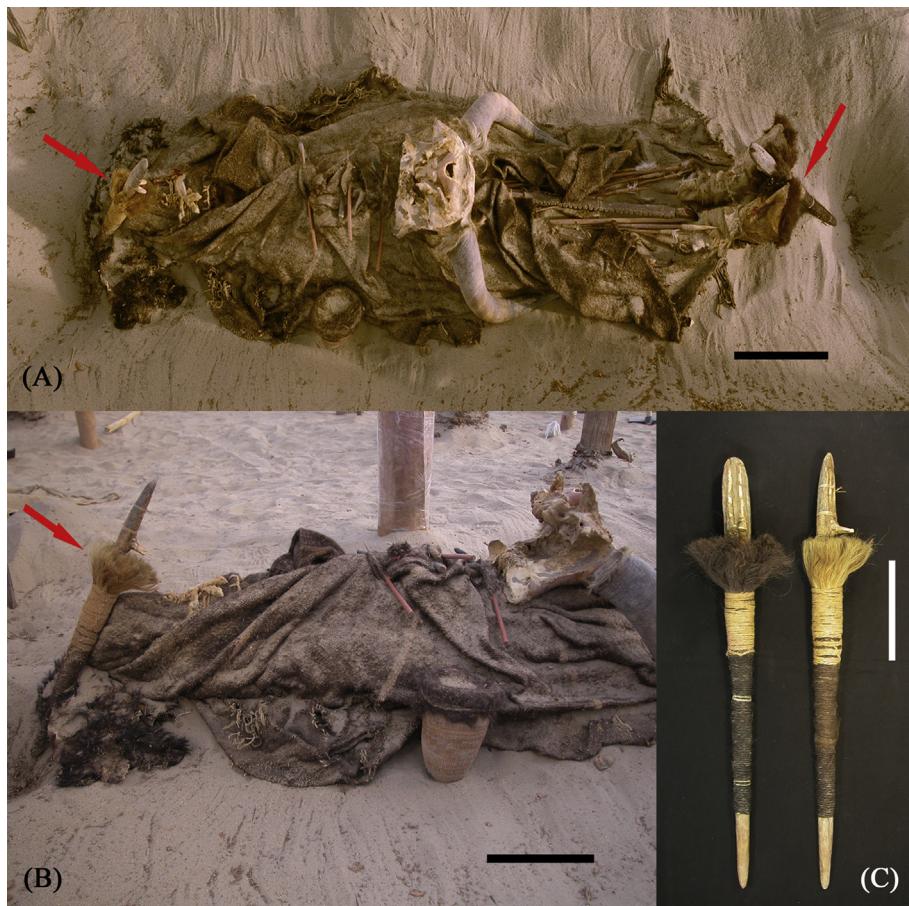


Fig. 2. The staffs discovered in tomb M24. (A) The front view of male mummy with his funerary objects (the wooden boat coffin was removed). The arrows point to the staffs standing at the occupier's head and feet. Scale bar = 30 cm (B) A detail side view of the staff near the head, as the arrow points. Scale bar = 20 cm. (C) An overall picture of the two staffs discovered. The right one named M24:9 was found at the occupier's head, while the left one named M24:10 found at the feet. Scale bar = 15 cm.

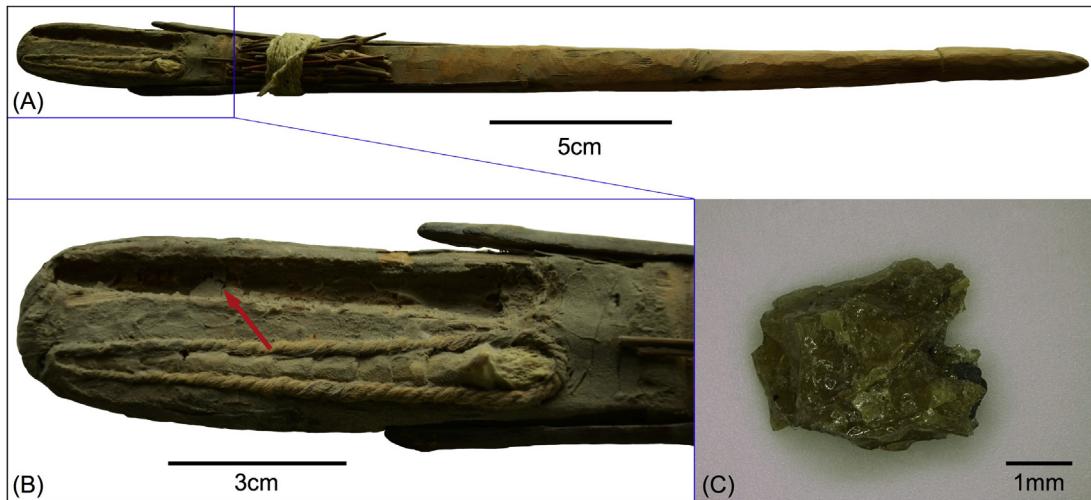


Fig. 3. Collected staff and analyzed adhesive. (A) The collected staff. The rectangle presents its upper section. (B) An enlarged view of the upper section. The arrow points to the groove where adhesive was collected. (C) The analyzed sample.

washed with water several times, immersed in the destaining solution (25% ethanol, 8% acetic acid) and slowly shaken overnight until the blue-stained protein area was visible.

- (3) In-gel digestion. The blue-stained protein area, as a whole on the gel slab, was cut into small particles of 1 mm^3 . Due to the smearing of protein down the gel, common in archaeological remains, the gel particles were pooled and collectively washed with distilled water three times. Then the particles were destained with 50% acetonitrile/25 mM NH_4HCO_3 , dried with 100% acetonitrile and alkylated in the dark with 50 mM iodoacetamide at room temperature for 30 min. After the supernatant was removed, gel particles were washed with 25 mM NH_4HCO_3 buffer twice, dried with 100% acetonitrile, and immersed in 12.5 ng/ μl trypsin solution in 25 mM NH_4HCO_3 . To ensure that the gel particles were covered with liquid. The digestion was incubated in the microwave oven at 850 W for 1 min and then the peptides were extracted with 100% acetonitrile. The extracted solution was vacuum dried and cryopreserved for further identification by MS.
- (4) LC/MS/MS. The digested sample was re-dissolved in 0.1% formic acid (buffer A) before MS analysis. Then it was analyzed by RP C18 capillary LC column from Michrom Bio-resources ($75\text{ }\mu\text{m} \times 150\text{ mm}$, $3\text{ }\mu\text{m}$). The eluted gradient was 5–30% buffer B (0.1% formic acid, 99.9% acetonitrile; flow rate, $0.3\text{ }\mu\text{l}/\text{min}$) for 30 min. The MS data were acquired on an LTQ Orbitrap Velos mass spectrometer using the following parameters: 20 data-dependent CID MS/MS scans per every full scan; full scans were acquired in Orbitrap at resolution 60,000; 35% normalized collision energy; internal mass calibration (445.120025 ion as lock mass with a target lock mass abundance of 0%); charge state screening (excluding precursors with unknown charge state or +1 charge state) and dynamic exclusion (exclusion size list 500, exclusion duration 30 s).
- (5) Database search. The MS/MS spectra were searched against the NCBI nr database (released 20,120,829, 20,093,899 sequences) using Mascot software version 2.4.1 (Matrix Science, UK). Trypsin was chosen as cleavage specificity with a maximum number of allowed missed cleavages of two. Carbamidomethylation (C) was set as a fixed modification, while deamidation (NQ) and oxidation (M) as variable modifications. The searches were performed using a peptide tolerance of 5 ppm and a product ion tolerance of 0.5 Da. For

further filtering the decoy search option was enabled and the peptide false discovery rate (FDR) was set to below 0.1% (the observed value was actually 0%). Protein identifications were accepted with two or more peptides and each peptide was matched with significance threshold $p < 0.05$ and ions score cut-off 30. In order to check the species-specificity of the sequences identified by Mascot software, each peptide sequence was submitted to the alignment process in the NCBI nr database using the protein Basic Local Alignment Search Tool (BLASTp tool) available online on the website of National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Where specified, separate database was compiled from the sequences of all bovine proteins (IPI_bovine, version_3.73, 30,403 sequences) and used for searching MS/MS spectra to obtain more related peptide sequences. In this case, the collagen post-transcriptional modifications (PTMs, i.e. hydroxylation of proline and lysine) were also set as variable modifications. Matched peptides were accepted with above filter. The peptides were BLAST searched again in the NCBI nr database to check the species-specificity of the sequences.

4. Results

4.1. FTIR analysis

The FTIR results are presented in Fig. 4. In comparison with literature data (Derrick et al., 1999; Zhou and Shen, 1997), it is possible to identify characteristic signals of an amide group ($-\text{N}(\text{H})-\text{C}=\text{O}-$). More specifically, the peak at 3411 cm^{-1} was assigned to N–H stretching vibration region, 1632 cm^{-1} to $\text{C}=\text{O}$ stretching vibration region, 1560 cm^{-1} to N–H bending vibration region, and 1405 cm^{-1} to C–N stretching region. The pattern of absorption peaks implies the presence of protein in the adhesive. Other peaks at 1126 , 637 and 618 cm^{-1} were probably attributed to asymmetric stretching and bending of sulfate (Periasamy et al., 2009), suggesting some inorganic components reserved. However, the overall FTIR spectrum of the sample did not match well with that of bone, which ruled out a possible contamination from the bone sculpture. To be specific, the characteristic peaks of hydroxyapatite were absent, including peaks at ~ 961 , 1012 and 1090 cm^{-1} corresponding to the symmetric (ν_1) and asymmetric (ν_3) stretching of phosphate, as well as peaks at ~ 557 and 600 cm^{-1}

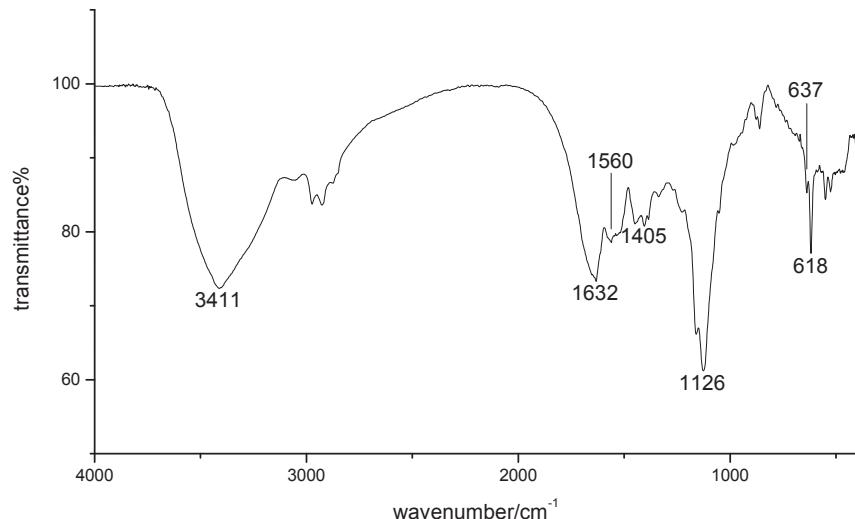


Fig. 4. FTIR spectrum of the sample.

to the bending (ν_4) of phosphate (Figueiredo et al., 2012; Huang et al., 2007).

4.2. Proteomic analysis

When searched against the NCBI nr database (released 20,120,829, 20,093,899 sequences), bovine collagen alpha-1(I) chain precursor and collagen alpha-2(I) chain precursor were identified as the main proteins except for disregarded human background proteins. 12 peptides from collagen $\alpha 1$ type I and 6 from collagen $\alpha 2$ type I were detected and the details of these peptides were displayed in Table S1. All the identified peptides were extracted and BLAST searched against the NCBI nr database to check the species-specificity of the sequences. In particular, sequence GPPGSAGSPGKDGLNGLPPIP^GPR (position 1141–1167) in collagen $\alpha 1$ type I and IGQPGAVGPAGIR (position 1066–1078) in collagen $\alpha 2$ type I have been assigned as bovine-specific peptides, confirming the bovine origin of collagen.

In order to obtain more related peptide sequences and corresponding detail information, a second Mascot search was conducted against the IPL_bovine database (version_3.73, 30,403 sequences) with the collagen PTMs (hydroxylation of proline and lysine) added as variable modifications. 43 peptides from collagen $\alpha 1$ type I and 37 from collagen $\alpha 2$ type I, including the 12 and 6 peptides identified in the first search, were detected and the details of these peptides were given in Table S2. After BLAST searches, the bovine-specific peptides were listed in Table 1, among which four bovine-specific peptides from collagen $\alpha 2$ type I (Table 1, entry d, e, g and h) have already been reported in previous work (Buckley et al., 2009; Buckley and Kansa, 2011; Dallongeville et al., 2011b, 2013). It can be noted that the hydroxylation of prolines occurred in all of the assigned peptides. Hydroxylysine (noted K, distinguished with lysine-K) was also found but with a lower frequency. As the variable hydroxylation may turn up at different sites of a single peptide, several MS/MS spectra could be assigned to the same sequence. As example, 8 MS/MS spectra were assigned to the peptide GPPGSAGSPGKDGLNGLPPIP^GPR (Table 1, entry a) with multiple modifications, whose detailed data including fragment ions are given in Fig. S1 (a–h). One of the MS/MS spectra assigned to GPPGSAGSPGKDGLNGLPPIP^GPR (the underlined residue representing the variable modification site; details given in Fig. S1 (d); ions score 45) is shown in Fig. 5, while Fig. 6 presents the MS/MS

spectrum assigned to IGQPGAVGPAGIR (Table 1, entry h; details given in Fig. S2 (c); ions score 46). In both figures, y and b represented the single charged mass fragments, y++ and b++ the double charged fragments, y0 and b0 the dehydrated fragments and y* and b* the deaminated fragments. Since the y and b ions had good continuity, the data is reliable.

The collagen triple helix composes of two $\alpha 1$ chains and one $\alpha 2$ chain which aggregate to form fibrils and fibers. Collagen type I (collagen $\alpha 1$ type I and collagen $\alpha 2$ type I) is the most abundant collagen in vertebrates and can be derived from skin, bones, tendons, etc. In addition, animal glue has been a kind of commonly used adhesive since ancient times, which is made by boiling the skin, bones or cartilaginous parts of mammals or fish, and composed mainly of collagen. Thus the obtained results suggest that the analyzed adhesive is a type of animal glue derived from bovine origins. Combing with the FTIR results, some inorganic components (sulfate) reserved might be the impurities of animal glue (especially skin glue) (Cai, 1991), owing to the preparation of the tissues before boiling (Pearson, 2003).

5. Discussion

5.1. Diverse utilizations of cattle in the Xiaohe Cemetery

Environmental analysis demonstrates that the Xiaohe people lived at a well-developed oasis surrounded by extensive desert (Li et al., 2013; Qiu et al., 2014), which was suitable for husbandry. Cattle and sheep were the main livestock in this area. Plenty of cattle related materials were discovered, including cattle skulls tied to the wooden grave markers and buried in the tombs, cattle hides wrapping the coffins, buried cattle ears, cowhide boots, bowstrings and threads made of cattle sinews, cattle dung, etc. (CRAIXAR, 2004, 2007). Proteomic analysis of residues in woven grass baskets and around mummified bodies also indicates that dairy foods were important components of the Xiaohe ancestors' diet (Liang et al., 2012; Yang et al., 2014). In summary, the Xiaohe people depended on cattle in many aspects of their lives. They used cattle in their religious practices, as a source of meat and milk, to provide leather and shelter, as a source of fertilizer or fuel (cow dung), and to arm themselves (bowstrings). Moreover, as indicated in this study, the leftover animal parts (bones, skin, tendons, etc.) were boiled down to make glues. This reveals the diverse utilizations of

Table 1

Bovine-specific peptides of collagen proteins identified in the sample.

Entry	Identified proteins	Species	Accession number	Specific peptides	Position	Variable hydroxylation sites
a	Collagen alpha-1(I) chain precursor	<i>Bos Taurus</i> (bovine)	NP_001029211.1	GPPGSAGSPGKDGLNGLPPIGPPGPR*	1141–1167	P2, P3, P9, K11, P18, P20, P23, P24, P26
b	Collagen alpha-2(I) chain precursor	<i>Bos Taurus</i> (bovine)	NP_776945.1	GAPGAIGAPGPAGANGDR	674–691	P3, P11
c				GAPGAIGAPGPAGANGDRGEAGPAGPAGPAGPR	674–706	P3, P9
d				SGETGASGPPGFVGEK	829–844	P9, P10
e				GYPGNAGPVGAAGAPGQGPVGPVGK	947–972	P3, P8, P15
f				HGNRGEPPAGAVGPAGAVGPR	973–994	P7
g				GEPGPAGAVGPAGAVGPR	977–994	P3
h				IGQPGAVGPAGIR*	1066–1078	P4
i				GSQGSQGPAGPPGPPGPPGPPGSGGYEFGFDGYR	1079–1116	P14, P15, P17, P18

* Indicates the bovine-specific peptides identified by both searches against the NCBI nr database and the IPI_bovine database.

cattle in the Xiaohe Cemetery, which provided not only meat, milk, hides, sinews and dung, but also leftover parts for manufacturing adhesive. Therefore, cattle played an extremely important role in the life of the Xiaohe people, confirming the significant impact of Eastern and Western communication as the cattle cultivated in the Xiaohe Cemetery likely originated from West Eurasian (Li, 2010).

5.2. Earliest evidence of adhesive use in China up to now

While the resin, gum, bitumen and tar, which could be gathered from nature or acquired through heating, were used as adhesive since the Paleolithic period and Middle Stone Age (Boëda et al., 2008; Charrié-Duhaut et al., 2013; Koller et al., 2001; Mazza et al., 2006; Wadley et al., 2009), animal glue arose later. The oldest animal glue discovered so far was occasionally used by Neolithic cave dwellers living southwest of the Dead Sea some 8000 years ago (Nissenbaum, 1997; Connan, personal communications). Animal glue was also the commonest adhesive in ancient Egypt since first recorded ca. 4000 BC (Koepff, 1985), and Egyptian carvings dating back 3300 years depicted glue preparation and use as well (Hull and Bangert, 1952; Pape, 1959). Concerning ancient Chinese literature, the historical book of "Zhou Li Kao Gong Ji", translated as the Records of Examination of Craftsman or Book of Diverse Crafts, which compiled science and technology in the Western Zhou dynasty (1046–771 BC) and should be written no later than the end of the

Spring and Autumn periods (770–476 BC), is the earliest literature specifically referring to animal glue, describing the color differences of several animal glues produced from deer, horse, cattle, mouse, fish and rhinoceros. Based on textual studies, Wang (1987) considered that the manufacture of animal glue in China has a history of ca. 3000 years. Scientific investigation of adhesive materials used in the Qin Shihuang's (the first emperor of the Qin dynasty) terracotta army (259–210 BC) (An, 2012), the Western Han dynasty terracotta army (206 BC–8 AD) (Wei et al., 2012), wall paintings at Kizil Grottoes (3rd century to 13th century) (Su et al., 2005), as well as Dunhuang mural paintings (5th century to 14th century) (Li, 1995), all confirm the application of animal glue. However, the research in this paper detected bovine glue used in prehistoric period. This is the earliest evidence of animal glue identified in China, which sheds light on adhesive development around 3500 years ago, nearly five hundred years earlier than previously thought.

Besides animal glue, other kinds of adhesives were discovered in China as well, such as beeswax and shellac as binding agents on Chinese turquoise-inlaid bronze swords dated to the East Zhou period (770–256 BC) (Cheng et al., 2008; Luo et al., 2012), urushi and linseed oil used on lacquer objects excavated from a Warring States Chu tomb (481–221 BC) (Wei et al., 2011), Chinese lacquer as binder in the Western Han dynasty polychrome terracotta jar (206 BC–23 AD) (Chiavari and Mazzeo, 1999), oxidized lacquer

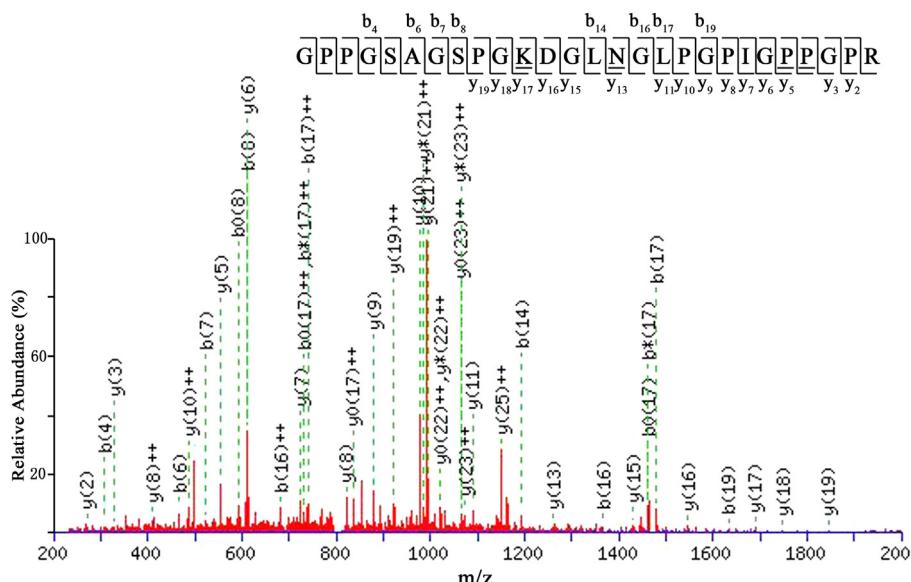


Fig. 5. MS/MS spectrum of the triply charged ion at $m/z = 819.0786$ and presenting the y and b fragments of the peptide sequence GPPGSAGSPGKDGLNGLPPIGPPGPR.(position 1141–1167) specific to bovine collagen $\alpha 1$ type I. Detailed data (peptide and fragment ions) were given in the Supporting Information (Fig. S1 (d)).

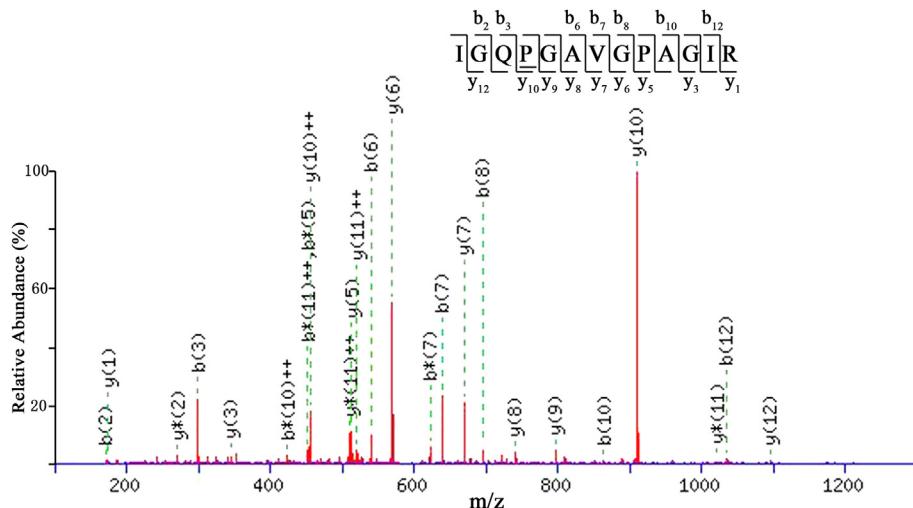


Fig. 6. MS/MS spectrum of the doubly charged ion at $m/z = 604.8411$ and presenting the y and b fragments of the peptide sequence **IGQPGAVGPAGIR** (position 1066–1078) specific to bovine collagen $\alpha 2$ type I. Detailed data (peptide and fragment ions) were given in the Supporting Information (Fig. S2 (c)).

mixed with tung oil as gilding adhesive on Buddha statue from the South Song dynasty (1127–1279 AD) (Hu et al., 2008), etc. Summarizing the adhesive materials discovered so far in various Chinese archaeological contexts, it is inferred that the analyzed sample from the Xiaohe Cemetery was the earliest adhesive investigated in China up to our knowledge.

6. Conclusions

More recently, proteomic methods have been utilized in archaeology and open new windows for residue analysis. The technique has been successfully applied in the identification of remains in archaeological pottery (Dallongeville et al., 2011a; Solazzo et al., 2008), binders in the paintings (Fremout et al., 2010; Leo et al., 2009), protein additives in building materials (Kuckova et al., 2009), etc. This work shows the efficiency of proteomic approaches on the identification of the prehistoric adhesive used as inlay material on a staff collected from the Xiaohe Cemetery, Xinjiang, China. Bovine-specific peptides of collagen type I were identified, suggesting that the adhesive was animal glue from bovine origin. This study reveals ca. 3500-year-old bovine glue used in ancient China, which is not only the earliest evidence of adhesive use in China, but also pushes back the history of Chinese animal glue to five hundred years earlier than previously thought. It also casts light on the life of the Xiaohe people, who raised cattle as one of the main livestock and depended on cattle in many aspects of their lives.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jas.2014.10.010>.

References

- Amat, E., 2011. An Exploration into the Social Life of Prehistoric Lopnur. College of History and Ethnology. Xinjiang Normal University, Urumqi, pp. 1–59 (in Chinese with English abstract).
- An, J., 2012. Analysis and Characterization of the Binding Media of Ancient Polychrome Artworks by MALDI-TOF-MS. College of Chemistry and Materials Science Northwest University, Xi'an, pp. 1–59 (in Chinese with English abstract).
- Boëda, É., Bonilauri, S., Connan, J., Jarvie, D., Mercier, N., Tobey, M., Valladas, H., Sakhel, H.a, 2008. New evidence for significant use of bitumen in Middle Palaeolithic technical systems at Umm el Tlel (Syria) around 70,000 BP. *Paléorient* 67–83.
- Buckley, M., Collins, M., Thomas-Oates, J., Wilson, J.C., 2009. Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 23, 3843–3854.
- Buckley, M., Kansa, S.W., 2011. Collagen fingerprinting of archaeological bone and teeth remains from Domuztepe, South Eastern Turkey. *Archaeol. Anthropol. Sci.* 3, 271–280.
- Cai, W., 1991. Animal glue. *Adhesion* 14–16 (in Chinese).
- Calvano, C., Van der Werf, I., Palmisano, F., Sabbatini, L., 2011. Fingerprinting of egg and oil binders in painted artworks by matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of lipid oxidation by-products. *Anal. Bioanal. Chem.* 400, 2229–2240.
- Chambery, A., Di Maro, A., Sangues, C., Severino, V., Tarantino, M., Lamberti, A., Parente, A., Arcari, P., 2009. Improved procedure for protein binder analysis in mural painting by LC-ESI/Qq-TOF mass spectrometry: detection of different milk species by casein proteotypic peptides. *Anal. Bioanal. Chem.* 395, 2281–2291.
- Charrié-Duhaut, A., Porraz, G., Cartwright, C.R., Igrelja, M., Connan, J., Poggenpoel, C., Texier, P.-J., 2013. First molecular identification of a hafting adhesive in the Late Howiesons Poort at diepkloof Rock shelter (Western Cape, South Africa). *J. Archaeol. Sci.* 40, 3506–3518.
- Cheng, X., Wu, S., Liu, Y., Zhou, L., 2008. Initial analysis on the adhesive in a bronze sword unearthed from East Zhou tombs in Zhoujiazhuang (Zhou Family Village), Shandong province. *Sci. Conservation Archaeol.* 20, 34–37 (in Chinese with English abstract).
- Chiavari, G., Mazzeo, R., 1999. Characterisation of paint layers in chinese archaeological relics by Pyrolysis-GC-MS. *Chromatographia* 49, 268–272.
- CRAIXAR, 2004. The excavation report of xiaohe Cemetery. In: RCCFAJU (Ed.), *Frontier Archaeology*, vol. 3. Science Press, Beijing, pp. 338–398 (in Chinese with English abstract).
- CRAIXAR, 2007. A brief excavation report on Xiaohe graveyard located in Luobupo, Xinjiang Autonomous Region. *Cult. Relics* 10, 4–42 (in Chinese with English abstract).
- Dallongeville, S., Garnier, N., Bernal Casasola, D., Bonifay, M., Rolando, C., Tokarski, C., 2011a. Dealing with the identification of protein species in ancient amphorae. *Anal. Bioanal. Chem.* 399, 3053–3063.
- Dallongeville, S., Koperska, M., Garnier, N., Reille-Taillefert, G., Rolando, C., Tokarski, C., 2011b. Identification of animal glue species in artworks using proteomics: application to a 18th century gilt sample. *Anal. Chem.* 83, 9431–9437.
- Dallongeville, S., Richter, M., Schäfer, S., Kühlenthal, M., Garnier, N., Rolando, C., Tokarski, C., 2013. Proteomics applied to the authentication of fish glue: application to a 17 th century artwork sample. *Analyst* 138, 5357–5364.

Derrick, M.R., Stulik, D., Landry, J.M., 1999. *Infrared Spectroscopy in Conservation Science*. Getty Publications.

Edwards, H.G.M., Stern, B., Burgio, L., Kite, M., 2009. Analysis of yellow “fat” deposits on Inuit boots. *Spectrochim. Acta A* 73, 561–565.

Fan, U., He, L., Thieme, C., Emmerling, E., 1996. Analysis and study of the pigments and binding media of the grand buddha grotto polychromy in bin county. *Dunhuang Res.* 140–153 (in Chinese with English abstract).

Figueiredo, M.M., Gamelas, J.A.F., Martins, A.G., 2012. Characterization of bone and bone-based graft materials using FTIR spectroscopy. In: Theophile, T. (Ed.), *Infrared Spectroscopy – Life and Biomedical Sciences*. InTech.

Fremout, W., Dhaenens, M., Saverwyns, S., Sanyova, J., Vandenabeele, P., Deforce, D., Moens, L., 2010. Tryptic peptide analysis of protein binders in works of art by liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta* 658, 156–162.

Fremout, W., Kuckova, S., Crhova, M., Sanyova, J., Saverwyns, S., Hynek, R., Kodicek, M., Vandenabeele, P., Moens, L., 2011. Classification of protein binders in artist's paints by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry: an evaluation of principal component analysis (PCA) and soft independent modelling of class analogy (SIMCA). *Rapid Commun. Mass Spectrom.* 25, 1631–1640.

Fremout, W., Sanyova, J., Saverwyns, S., Vandenabeele, P., Moens, L., 2009. Identification of protein binders in works of art by high-performance liquid chromatography-diode array detector analysis of their tryptic digests. *Anal. Bioanal. Chem.* 393, 1991–1999.

Hu, D., Xue, Y., Wang, J., Zhang, H., 2008. An analytical study of gilding and adhesive materials on the one-thousand-hand Buddha at Dazu Grotto, Baoding Mountain, Chongqing. *Sci. Conservation Archaeol.* 20, 44–51 (in Chinese with English abstract).

Huang, C.M., Zhang, Q., Bai, S., Wang, C.S., 2007. FTIR and XRD analysis of hydroxyapatite from fossil human and animal teeth in Jinsha Relict, Chengdu. *Guang pu xue yu guang pu fen xi = Guang pu* 27, 2448–2452 (in Chinese with English abstract).

Hull, W.Q., Bangert, W.G., 1952. Animal glue. *Ind. Eng. Chem.* 44, 2275–2284.

Koepff, P.J., 1985. History of industrial gelatine production (with special reference to photographic gelatine). In: Ammann-Brass, H., Pouradier, J. (Eds.), *Photographic Gelatin, Proceedings of the Fourth IAG Conference*. International Working Group for Photographic Gelatin, Fribourg, pp. 3–31.

Koller, J., Baumer, U., Mania, D., 2001. High-tech in the Middle Palaeolithic: neandertal-manufactured pitch identified. *Eur. J. Archaeol.* 4, 385–397.

Kuckova, S., Crhova, M., Vankova, L., Hnizda, A., Hynek, R., Kodicek, M., 2009. Towards proteomic analysis of milk proteins in historical building materials. *Int. J. Mass Spectrom.* 284, 42–46.

Leo, G., Cartechini, L., Pucci, P., Sgamellotti, A., Marino, G., Birolo, L., 2009. Proteomic strategies for the identification of proteinaceous binders in paintings. *Anal. Bioanal. Chem.* 395, 2269–2280.

Li, C., 2010. Molecular Genetic Analysis of Ancient Remains from Xiaohe Cemetery. College of Life Science, Jilin University, Changchun, pp. 1–132 (in Chinese with English abstract).

Li, C., Li, H., Cui, Y., Xie, C., Cai, D., Li, W., Mair, V.H., Xu, Z., Zhang, Q., Abuduresule, I., 2010. Evidence that a West-East admixed population lived in the Tarim Basin as early as the early Bronze Age. *BMC Biol.* 8, 15.

Li, J., Abuduresule, I., Hueber, F.M., Li, W., Hu, X., Li, Y., Li, C., 2013. Buried in sands: environmental analysis at the archaeological site of Xiaohe Cemetery, Xinjiang, China. *Plos One* 8, e68957.

Li, S., 1995. The quantitative analysis of the binding media in Dunhuang wall paintings. *Dunhuang Res.* 29–46 (in Chinese).

Liang, Y., Yang, Y., Abuduresule, I., Li, W., Li, X., Wang, C., 2012. Proteomic analysis of residues in grass basket excavated from Xiao-He graveyard. *Sci. Conservation Archaeol.* 24, 81–85 (in Chinese with English abstract).

Liu, X., 2009. *The Prehistoric Religious Studies in Xinjiang*. Ethnic Publishing House, Beijing (in Chinese).

Luo, W., Li, T., Wang, C., Huang, F., 2012. Discovery of Beeswax as binding agent on a 6th-century BC Chinese Turquoise-inlaid Bronze sword. *J. Archaeol. Sci.* 39, 1227–1237.

Mazza, P.P.A., Martini, F., Sala, B., Magi, M., Colombini, M.P., Giachi, G., Landucci, F., Lemorini, C., Modugno, F., Ribechni, E., 2006. A new Palaeolithic discovery: tar-hafted stone tools in a European Mid-Pleistocene bone-bearing bed. *J. Archaeol. Sci.* 33, 1310–1318.

Mitkidou, S., Dimitrakoudi, E., Urem-Kotsou, D., Papadopoulou, D., Kotsakis, K., Stratis, J.A., Stephanidou-Stephanatou, I., 2008. Organic residue analysis of Neolithic pottery from North Greece. *Microchim. Acta* 160, 493–498.

Modugno, F., Ribechni, E., Colombini, M.P., 2006. Chemical study of triterpenoid resinous materials in archaeological findings by means of direct exposure electron ionisation mass spectrometry and gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* 20, 1787–1800.

Nissenbaum, A., 1997. 8000 Years Collagen from Nahal Hemar cave. *Archaeol. Nat. Sci.* 5–9.

Pape, M.E., 1959. Costs and inventory values in the glue industry. *Account. Rev.* 34, 52–58.

Pearson, C.L., 2003. Animal glues and adhesives. In: Pizzi, A., Mittal, K.L. (Eds.), *Handbook of Adhesive Technology*, second ed. Marcel Dekker, Inc., New York.

Periasamy, A., Muruganand, S., Palaniswamy, M., 2009. Vibrational studies of Na₂SO₄, K₂SO₄, NaHSO₄ and KHSO₄ crystals. *Rasayan J. Chem.* 2, 981–989.

Pike, R.A., 2014. Adhesive. Encyclopedia Britannica Online, Retrieved 27 January, 2014, from <http://www.britannica.com/EBchecked/topic/5823/adhesive>.

Qiu, Z., Yang, Y., Shang, X., Li, W., Abuduresule, Y., Hu, X., Pan, Y., Ferguson, D.K., Hu, Y., Wang, C., 2014. Paleo-environment and paleo-diet inferred from Early Bronze Age cow dung at Xiaohe Cemetery, Xinjiang, NW China. *Quat. Int.* 349, 167–177.

Regert, M., 2004. Investigating the history of prehistoric glues by gas chromatography–mass spectrometry. *J. Sep. Sci.* 27, 244–254.

Scott, D.A., Warmlander, S., Mazurek, J., Quirke, S., 2009. Examination of some pigments, grounds and media from Egyptian cartonnage fragments in the Petrie Museum, University College London. *J. Archaeol. Sci.* 36, 923–932.

Solazzo, C., Fitzhugh, W.W., Rolando, C., Tokarski, C., 2008. Identification of protein remains in archaeological potsherds by proteomics. *Anal. Chem.* 80, 4590–4597.

Su, B., Sinkei, T., Hu, Z., Li, Z., 2005. HPLC analysis of binding medium of pigments of Wall painting at kizil Grottoes. *Dunhuang Res.* 57–61 (in Chinese with English abstract).

Wadley, L., Hodgskiss, T., Grant, M., 2009. Implications for complex cognition from the hafting of tools with compound adhesives in the Middle Stone Age, South Africa. *Proc. Natl. Acad. Sci.* 106, 9590–9594.

Wang, Y., 1987. The origin of animal glue. *Sci. Technol. Gelatin* 7, 148–151 (in Chinese).

Wei, S., Ma, Q., Schreiner, M., 2012. Scientific investigation of the paint and adhesive materials used in the Western Han dynasty polychromy terracotta army, Qingzhou, China. *J. Archaeol. Sci.* 39, 1628–1633.

Wei, S., Pintus, V., Pittard, V., Schreiner, M., Song, G., 2011. Analytical characterization of lacquer objects excavated from a Chu tomb in China. *J. Archaeol. Sci.* 38, 2667–2674.

Yang, L., Wang, L., Huang, J., Ma, T., 2010. Identification of binding media by amino acid analysis from a theater in J iayuguan pass. *J. Lanzhou Univ. Nat. Sci.* 46, 125–128 (in Chinese with English abstract).

Yang, X., Wan, Z., Perry, L., Lu, H., Wang, Q., Zhao, C., Li, J., Xie, F., Yu, J., Cui, T., Wang, T., Li, M., Ge, Q., 2012. Early millet use in northern China. *Proc. Natl. Acad. Sci. U. S. A.* 109, 3726–3730.

Yang, Y., Shevchenko, A., Knaust, A., Abuderesule, I., Li, W., Hu, X., Wang, C., Shevchenko, A., 2014. Proteomics evidence for kefir dairy in Early Bronze Age China. *J. Archaeol. Sci.* 45, 178–186.

Zhang, Y., Weng, Y., Yao, L., Zhang, J., Zhou, Y., Fang, F., Cui, W., 2011. Identification and analysis of starch granules on the surface of the slabs from Peiligang site. *Quat. Sci.* 31, 891–899 (in Chinese with English abstract).

Zhou, R., Shen, Y., 1997. Study on secondary structures of a protein by infrared spectra. *J. East China Univ. Sci. Technol.* 23, 422–425 (in Chinese with English abstract).